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PRINCIPAL INVESTIGATOR: Ian N. Jongewaard, Ph.D.

RECIPIENT ORGANIZATION: The Burnham Institute
La Jolla, CA 92037

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FOREWORD

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INTRODUCTION

Integrins are non-covalently associated $\alpha\beta$ heterodimers that mediate cell adhesion and cell migration. Integrins have key roles in thrombosis, atherogenesis, tumor dissemination, angiogenesis and viral infection (1-4). Integrins direct many cell-matrix contacts and also transmit vital cellular signals (5,6). The subject of this study is the integrin $\beta 5$ subunit, which forms a heterodimer with αv and can bind to the RGD tripeptide motif (7). The $\alpha v\beta 5$ integrin is known to be a cell surface receptor for many adenoviruses (4,8) and to play a crucial role in angiogenesis (9). The $\beta 5$ subunit is most closely related to the integrin $\beta 3$ subunit (10,11). The two proteins are 56% identical at the amino acid level and the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins share many of the same ligands (7,12).

The integrity of the integrin $\alpha\beta$ heterodimer is thought to be essential for expression on the cell surface and for ligand binding function (13-16). Considerable evidence also demonstrates that the cytoplasmic tails of the integrin $\alpha\beta$ heterodimer interact to control signal transduction (17-20). For example, deletions within either the cytoplasmic tail of the α or β subunit can change the affinity of the integrin for ligand (20), suggesting that the interaction between the two tails places a conformational constraint on the receptors ectodomain. A more in-depth analysis has indicated that a salt bridge may physically connect the two cytoplasmic tails (21). When this salt bridge is disrupted, the affinity state and signaling properties of the integrin can be altered. Other data, however, suggests that the cytoplasmic tails of the integrin α and β subunits may act autonomously. For example, yeast two-hybrid screens have identified proteins that will bind to the cytoplasmic tails of the integrin β subunits (22,23), and affinity chromatography has shown that calreticulin can bind to a conserved motif within the cytoplasmic tails of the α subunits (24).

Other studies have shown that when β subunit tails are expressed as fusion proteins with unrelated membrane proteins, they can direct the localization of those proteins to focal adhesion sites (25). Similarly, fusion proteins containing an integrin cytoplasmic tail have been shown to negatively regulate cell adhesion (26) and to interfere with signaling (27). These studies have been taken as an indication that the cytoplasmic tail of the β subunit can function independently. Despite the evidence to indicate that the α and β subunit cytoplasmic domains may have autonomous functions, no data have indicated how these domains could be displayed in the absence of their partner in a physiologic setting.

Here, evidence is presented challenging the doctrine that integrins exist solely as $\alpha\beta$ heterodimers. The integrin $\beta 5$ subunit is subject to a series of novel and highly specific alternative splices. Surprisingly, the splice variants are expressed on the cell surface as single-subunit integrins. Each splice variant contains a transmembrane domain and a cytoplasmic tail. Thus, the variants of $\beta 5$ represent the first identification of an autonomously expressed integrin subunit. This finding adds another level of diversity to the integrin protein family; and also suggests a potential physiologic means of modulating cell adhesion, cellular signaling and integrin subunit composition.

EXPERIMENTAL METHODS AND PROCEDURES

cDNA cloning and DNA sequence analysis. Total RNA was isolated from MDA-MB-435 cells using RNazol (28). RNA was converted to first strand cDNA using reverse transcriptase and then used as template in PCR amplification with oligonucleotide primers designed to amplify overlapping portions of the known $\beta 5$ cDNA. The amplified DNA bands were analyzed by gel electrophoresis. Bands that were smaller than the predicted size for a given primer pair were cloned into the pBluescript II vector (Stratagene) by taking advantage of unique restriction sites that were engineered into each of the primers. The complete DNA sequence of all clones was verified by sequencing from both strands (29).

PCR analysis of alternative splice expression. Total RNA was isolated from MDA-MB-435 cells and human foreskin fibroblasts as described above. This RNA was converted to first strand cDNA using reverse transcriptase and then used for PCR amplification. PCR amplification of specific splice variants was accomplished by using a single reverse primer for all of the splice variants. This "universal" reverse primer was used in combination with a forward primer specific to each splice variant. The universal reverse primer for all amplifications of the $\beta 5$ splice variants was: 5'-CCTGGTGCCCAGGTAGCCGGGGCTG; The forward primer for the $\beta 5$ D/H splice variant was: 5'-TCCAGGCAGCCGTCTGCAAGAGTA-3'; The forward primer for the $\beta 5$ E/H splice variant was: 5'-ACACTGCATCCAACCAGATGAGTA-3'; The forward primer for the $\beta 5$ G/I splice variant was: 5'-TGATTATTAATGCATACAATGCAT-3'; The universal reverse primer for all amplifications of the $\beta 3$ sequence was: 5'-GACGGGGCTGACCCTCTCGGGGGCTG-3'; The forward primer used in an

attempt to detect a $\beta 3$ D/H splice variant was: 5'-TGCAGGCTACAGTCTGTGATAAAA-3'; The forward primer used in an attempt to detect a $\beta 3$ E/H splice variant was: 5'-ACTCTGCCTCCACTACCATGAAAA-3'; The forward primer used in an attempt to detect a $\beta 3$ G/I splice variant was: 5'-TCATTGTTGATGCTTATGGGGTGA-3'). Amplified DNA fragments were visualized using agarose gel electrophoresis and ethidium bromide staining.

Subcloning and transfection of 293 cells with the splice variants. The overlapping fragments of $\beta 5$ cDNA generated while cloning the portions of the cDNA that are alternatively spliced, were pieced together to produce full length cDNA and were used as a backbone for subcloning in the alternatively spliced exons. Each of these cDNA were then subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Human embryonic kidney 293 cells were transfected with each of the splice variants using cationic lipid transfer (DOTAP, Boehringer Mannheim) as outlined in the literature accompanying the product. Following selection with G418 (200ug/ml, Life Technologies), stable cell lines were used for the surface expression and subunit association experiments.

Cell Surface iodination and immunoprecipitation. The immunoprecipitations of ^{125}I -labeled cells were carried out essentially as described (30). Protein bands were visualized by SDS PAGE on 7.5% acrylamide gels followed by autoradiography. The amount of each subunit remaining after immunodepletion was quantified from the intensity of the immunoprecipitated bands using a Bio Rad GS 525 Molecular Imager System.

Antibody production. Polyclonal antiserum was raised against an 11 amino acid peptide which corresponds to the splice junction at the boundary of exons D and H of $\beta 5$ (protein sequence = gln-ala-ala-val-cys-lys-ser-ile-arg-ser-lys). Antisera was raised by conjugating the peptide to Keyhole Limpet Hemocyanin and inoculating rabbits with the conjugated antigen as described(31). Attempts to obtain polyclonal antisera against the other splice variants of $\beta 5$ have been unsuccessful to date.

RESULTS AND DISCUSSION

Identification of Alternatively Spliced forms of the Integrin $\beta 5$ Subunit in Breast Carcinoma Cells

This study was initiated after it was observed that immunoprecipitation of the integrin $\beta 5$ subunit from MDA-MB-435 breast carcinoma cells yielded multiple protein bands. Both a monoclonal antibody and a polyclonal antiserum raised against the $\beta 5$ subunit immunoprecipitated a broad protein band with a mass substantially lower than wild-type $\beta 5$ (Figure 1). This additional band represents a significant portion of the protein immunoprecipitated with antibodies against $\beta 5$ (~30%). We hypothesized that this broad band is related to $\beta 5$. A reverse transcriptase polymerase chain reaction (rtPCR) cloning strategy was used to amplify cDNA's from the MDA-MB-435 cells which encode the $\beta 5$ -like protein. Three PCR products of aberrant size were amplified and cloned. Each clone is an alternatively spliced form of $\beta 5$ (Figure 2). All three alternatively spliced forms of $\beta 5$ are lacking exons in the amino-terminal one third of the protein. The splice variants of $\beta 5$ are designated D/H, E/H and G/I, to connote the exons which are contiguous within the splice. The D/H variant, for example, is lacking exons E, F and G placing the D and H exons in juxtaposition.

The mRNA Encoding the Integrin $\beta 5$ Subunit is Alternatively Spliced but mRNA Encoding $\beta 3$ is not

A PCR strategy was used to determine whether the alternative splices of $\beta 5$ are expressed in a non-transformed cell line and to determine whether the closely related integrin $\beta 3$ subunit is similarly spliced. Each splice variant of $\beta 5$ is present in both MDA-MB-435 cells and in normal human foreskin fibroblasts (Figure 3) indicating that cellular transformation is not necessary for the

splicing of this mRNA. The integrin $\beta 3$ subunit, which is also expressed by both cell types, is not spliced (Figure 3). Consequently, the novel splicing mechanism can distinguish the mRNA's encoding $\beta 3$ and $\beta 5$, even though they are highly related (11). Additional PCR reactions proved that the mRNA encoding each splice variant is mature (polyadenylated) and is also contiguous with the remainder of the wild-type $\beta 5$ sequence (not shown).

Variant Forms of the $\beta 5$ Subunit are Expressed on the Cell Surface as Single Subunit Integrins

Each spliced form of $\beta 5$ is translated to protein and expressed on the cell surface. Human embryonic kidney 293 cells, which express only low levels of endogenous $\beta 5$, were transfected with cDNA's encoding each of the splice variants. The transfected 293 cells expressed the alternatively spliced forms of $\beta 5$ on the cell surface (Fig. 4A, arrows). The $\alpha v\beta 5$ heterodimer that is endogenously expressed by the 293 cells co-immunoprecipitates in these experiments. Each spliced version of $\beta 5$ is lacking a domain of the β -subunit thought to be essential for $\alpha\beta$ association (15,32,33); therefore, we examined whether the splice variants associate with αv . To distinguish the expression of the new proteins as autonomous β -subunits from their potential association with αv , lysates from the transfected 293 cells were immunodepleted of αv -associated protein. Subsequently, the lysate was immunoprecipitated with antibodies against $\beta 5$. Immunodepletion of the αv integrin did not reduce the level of any of the alternatively spliced forms of $\beta 5$ on the cell surface (Fig. 4B). This immune-depletion did, however, deplete the endogenous $\beta 5$. Therefore, unlike $\beta 5$, which pairs with αv , each variant of $\beta 5$ is expressed on the cell surface as a single subunit integrin. To independently confirm that the splice variants are expressed autonomously, immunoprecipitations were performed

with an antibody raised against a synthetic peptide corresponding to the protein sequence at the D/H splice junction. As shown in Fig. 4C, this polyclonal antibody immunoprecipitated a single band from the MDA-MB-435 cells, with a mass corresponding to the predicted mass of the D/H variant. Thus, the D/H splice variant of $\beta 5$ is expressed endogenously by the MDA-MB-435 line and is displayed on the cell surface without associating with αv . Attempts to raise antibodies against the other splice junctions have been unsuccessful.

Prior to this report it was believed that all integrins existed as non-covalently associated $\alpha\beta$ heterodimers. However, the data presented here demonstrate the existence of three splice variants of the integrin $\beta 5$ subunit that do not associate with any α -subunit partner. Each splice eliminates a segment of the ectodomain of $\beta 5$ while maintaining the protein reading frame. Therefore, all three of the splice variants have a modified ectodomain, but intact transmembrane domains and cytoplasmic tails. Thus, the mRNA splicing enables the unique display of the $\beta 5$ cytoplasmic tail in a form that is uncoupled from a subunit association and also from normal ligand binding function.

The function of the spliced version of $\beta 5$ are not immediately evident, but prior study suggests that they could modulate integrin-dependent signaling. Several reports indicate that a disruption in the integrity of the integrin $\alpha\beta$ heterodimer, particularly any interactions within the cytoplasmic tails, could regulate both "inside out" and "outside-in" signal transduction. For example, the autonomous expression of a cytoplasmic domain from an integrin β subunit is known to regulate integrin mediated signaling (27). When expressed as a fusion protein with the IL-2 receptor, the cytoplasmic domain of integrin $\beta 3$ subunit is sufficient to stimulate integrin-specific tyrosine phosphorylation (19). Similarly, autonomously expressed β -subunit cytoplasmic tails can act as

trans-dominant inhibitors of integrin function because of their ability to induce cellular de-adhesion (26) and inhibit "inside-out" signal transduction (27). The data presented here, showing that alternative splices of $\beta 5$ are displayed as single subunit integrins, suggest that alternative splicing of the message encoding $\beta 5$ may be a physiologic means of displaying an autonomous integrin β -subunit positioned at the cell surface.

CONCLUSIONS

Three novel alternative splice forms of the $\beta 5$ integrin subunit have been identified in human breast carcinoma cells. These alternative splice forms appear to be expressed on the cell surface as single subunits.

In the four months of research activity covered by this progress report (July 1, 1997 to October 31, 1997), little progress has been made in testing the hypothesis that the alternative splice variants of $\beta 5$ can modulate the adhesive capacity and the migratory rate of tumor cells. This is not for lack of trying. Each of the alternative splice variants have been subcloned into a mammalian expression vector (pIND, Invitrogen, Carlsbad, CA). In this vector, expression of the alternative splice variants is under the control of an ecdysone inducible promoter. Human embryonic kidney 293 cells have been transfected with these constructs and G418/Zeocin double resistant cell populations have been selected. Unfortunately, inducible expression of the alternative splice variants within these populations has not been demonstrated.

Determination of the abundance and localization of the alternative splice forms has been problematic and hampered by the lack of subunit specific antibodies. We have been able to produce only one subunit specific polyclonal antiserum. This antiserum recognizes the D/H splice variant and works very well in immunoprecipitation of that form of $\beta 5$. Unfortunately, this antiserum has not been used successfully in immunolocalization, Western blotting, or FACS analysis (multiple attempts have been made at each of these types of analyses). Until additional specific antibodies are available, determination of the abundance and localization of the alternative splice variants will be difficult at best.

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Figure 1 Immunoprecipitation of multiple forms of $\beta 5$ from MDA-MB-435 cells. The integrin $\beta 5$ subunit was immunoprecipitated from MDA-MB-435 breast carcinoma cells using the monoclonal antibody 15F11, which recognizes the $\beta 5$ subunit (34), and with a polyclonal antibody raised against the cytoplasmic tail of $\beta 5$, designated as $\beta 5T$ (35). Arrows indicate the positions of αv , $\beta 5$, and a protein of lower mass which we hypothesized was related to $\beta 5$. This experiment was repeated at least three times, each yielding the same result.

15F11

β_5 T



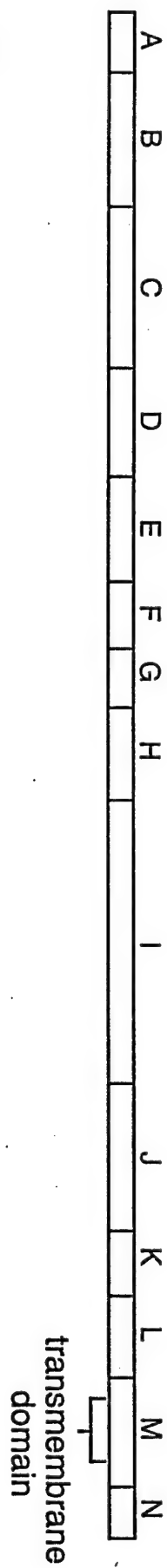
← α_v

← β_5

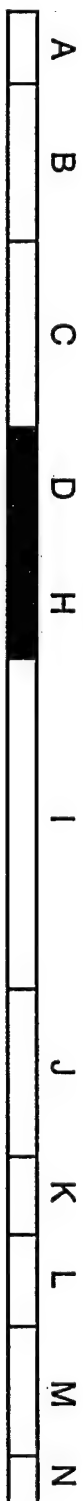
← $\beta_5?$

Figure 2. Exon Map of the Alternatively Spliced Forms of $\beta 5$. PCR cloning was used to identify three novel mRNA's encoding $\beta 5$ -related sequences from the MDA-MB-435 cells. The nucleotide sequences determined by dideoxy sequencing showed that each clone contained a discontinuous segment of $\beta 5$. A comparison of each clone with the known genomic structure of both the integrin $\beta 1$ and $\beta 3$ subunits (36) revealed that the novel cDNA's encoded alternatively spliced forms of $\beta 5$. Each clone has exon junctions identical to those reported from the genomic sequences of $\beta 1$ and $\beta 3$. The deduced exon structure of each new form of $\beta 5$ is shown.

Full Length Beta 5



D/H



E/H



G/I



Figure 3. The $\beta 5$ Splicing Mechanism Bypasses the mRNA Encoding $\beta 3$. A reverse transcriptase PCR amplification strategy was established to detect the mRNA encoding the $\beta 5$ splice variants and to determine whether the closely related $\beta 3$ subunit is similarly spliced. These amplification reactions were designed to distinguish the spliced mRNA from wild-type mRNA. Primers for the $\beta 5$ splices were designed to hybridize across the 5' end of each splice junction, and to amplify only the splice variants. Similar primers were used to identify analogous splicing within the $\beta 3$ message, although the primers used to amplify a D/H splice variant of $\beta 3$ were also designed to amplify a segment of the full-length $\beta 3$ (732 base pairs) as an internal control. mRNA samples from the both MDA-MB-435 breast carcinoma cells and from normal human foreskin fibroblasts were analyzed. The predicted sizes of the amplified fragments are; 384 base pairs for the D/H and E/H splice variants and 249 base pairs for amplification of the G/I splice variant. All of the PCR analysis was repeated at least three times with equivalent results.

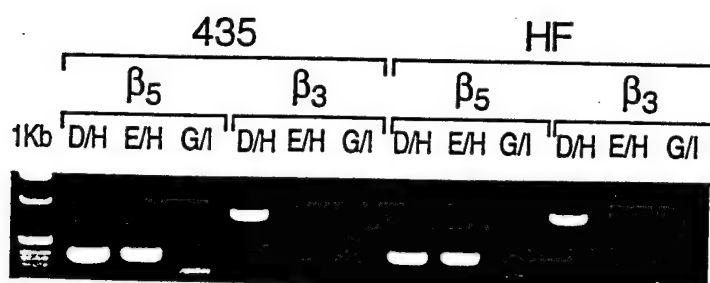
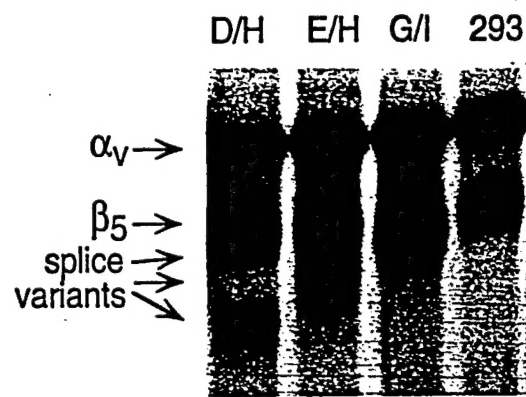


Figure 4. The Variant Forms of $\beta 5$ are Expressed on the Cell Surface as Single-subunit Integrins. (A), The ability of each splice variant of $\beta 5$ to be expressed as protein was determined by transfecting kidney 293 cells with expression vectors containing the cDNA encoding each splice variant. The 293 cells were chosen because they express the αv subunit, but only low levels of endogenous $\beta 5$. The cell lines expressing each variant were surface labeled with ^{125}I Na and then immunoprecipitated as described (30). Arrows indicate the position of each splice variant and endogenous $\beta 5$ on the polyacrylamide gel. (B), To determine whether the spliced versions of $\beta 5$ associate with αv , the αv subunit was immunodepleted from surface labeled protein samples prior to immunoprecipitation. The αv specific monoclonal antibody L230 (37) was used to immunodeplete cell lysates from the three transfected cell lines. Immunodepletion was followed by immunoprecipitation with 15F11 to precipitate any remaining wild-type $\beta 5$ or $\beta 5$ splice variant. The cell lines from which the lysates were prepared are listed above the graph and the individual subunits from each of the cell lines are listed below. Each depletion was repeated at least three times with equivalent results. (C), The D/H splice variant can be immunoprecipitated as a single subunit from MDA-MB-435 cells. Antisera raised against the D/H peptide was used to immunoprecipitate

lysates from radiolabeled MDA-MB-435 cells. lane 1, immunoprecipitation with the anti-peptide antibody; lane 2, immunoprecipitation with the anti-peptide antibody in the presence of 10 mg/ml D/H peptide; lane 3, immunoprecipitation with the anti-peptide antibody in the presence of 10 mg/ml unrelated peptide (with sequence ser-pro-ala-ser-ser-phe-his-val-leu-arg-ser-leu). This experiment was repeated twice with the same results.

A



B

**Percent of integrin subunit
remaining after immunodepletion**

